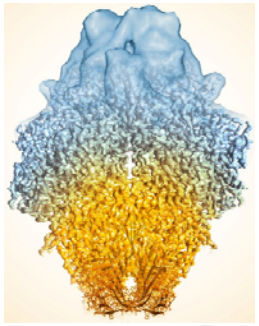
Sample preparations for structural studies by TEM



β-Galactosidase
Subramaniam Lab

Olivier Lambert
o.lambert@cbmn.u-bordeaux.fr

Ernst Ruska **(1906-1988) built the first TEM in 1931 (mag 17.4) (Nobel prize 1986)**

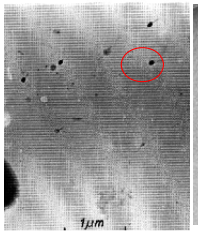
1931 — 1970 — 1990 — 2000 — 2008

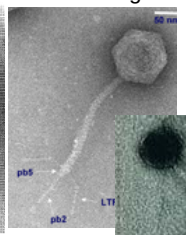
Bacteriophages. (H Ruska, 1941-1942)

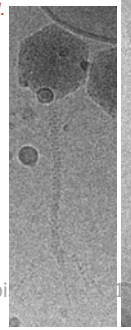
Negative staining

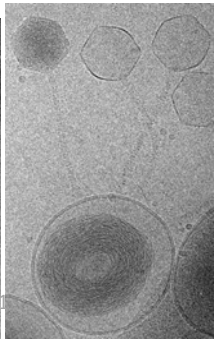
1982
Vitrication technology
Dubochet *et al.*

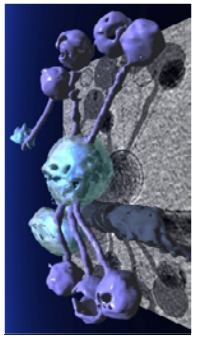
cryoTEM



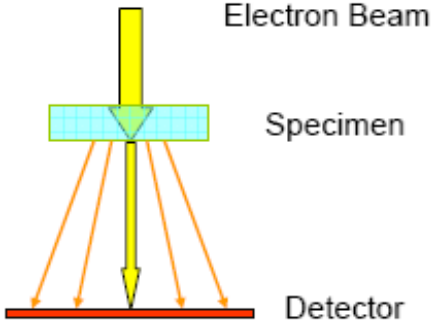








Biological samples in the electron microscope environment

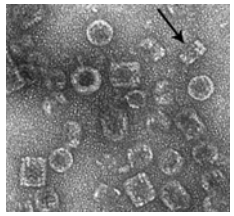


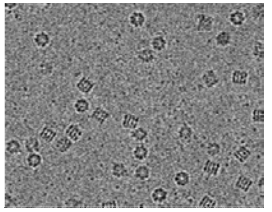
They have to: - be thin enough

They have to: - resist to the vacuum and to the electrons
- stay in their native form

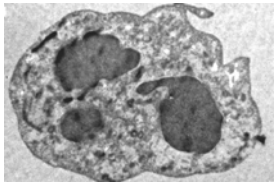
Preparation regarding the size of biological samples

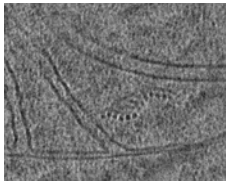
	Dehydrated / stained specimen	**Frozen hydrated/ unstained specimen**
Thin Specimen :	**negative staining**	**freeze-plunging** Cryo-electron microscopy





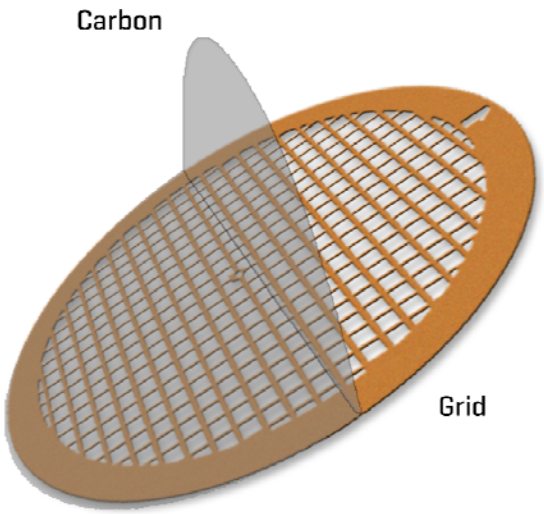
Thick Specimen :	**Plastic section**	**CEMOVIS, cryosection**



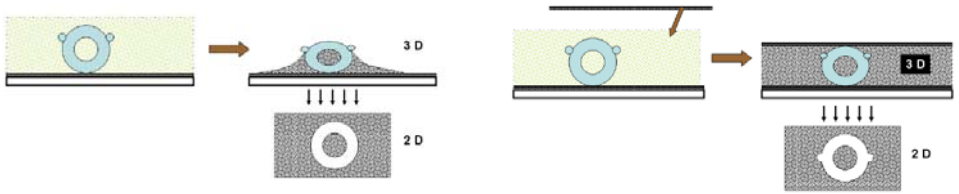


Negative Staining

Support : grid coated with thin amorphous carbon



Negative Staining :Principle

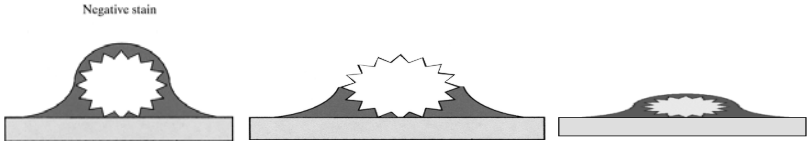


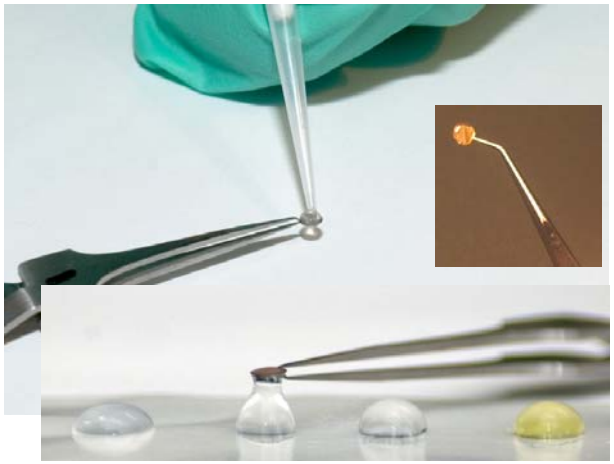
Advantages :

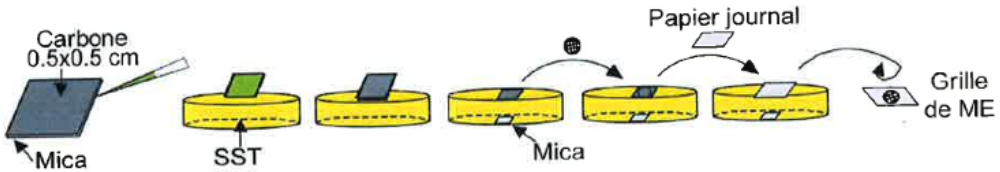
Fast
Small amount of protein (concentration 0.01-0.1mg/ml, few µl)
Small proteins are visible
High contrast

Drawbacks :

Low resolution (15 Å)
Flattening and drying
Footprint of the sample in the heavy atom
Artefacts due to the stain







Valentine et al, 1968. Biochemistry 7:2143-52

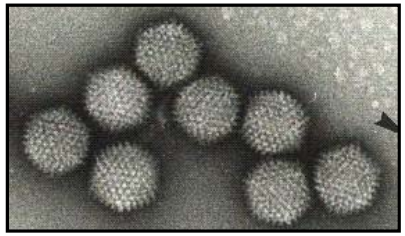
Negative Staining: Several stains available

Stains:
Uranyl formate (finer grain, but unstable)
Uranyl acetate (coarser grain but stable)
-High contrast and radiation resistant
-- but acidic (pH 4)
-It also function as fixative !

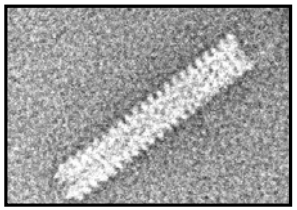
Sodium silicotungstate
Sodium phosphotungstate (thin layer of stain)
Ammonium molybdate (thick layer of stain)
Aurothioglucose
-- neutral pH
-- but lower contrast and less radiation-resistant

Ohi et al., 2004 Biol. Proced. Online 6: 23-34

Negative Staining : Examples of macromolecular assemblies

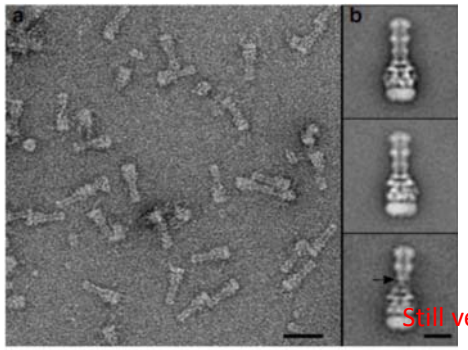


Adenovirus
(100 nm)
Courtesy of G Schohen

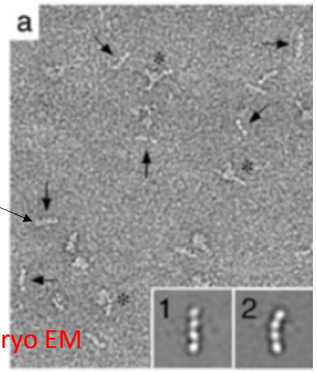


Measles nucleocapsid Pitch 5 nm
Courtesy of G Schohen

Tripartite efflux system (TSS1-like)
Daury et al 2016, Nature comm



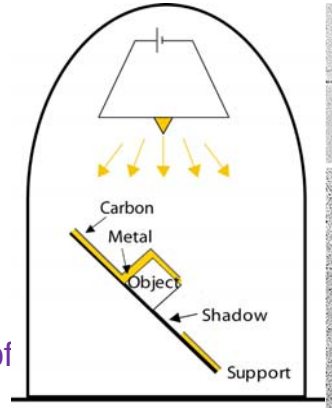
Integrin α5β1 in complex with fibronectin.
Takagi J,*EMBO J* 2003

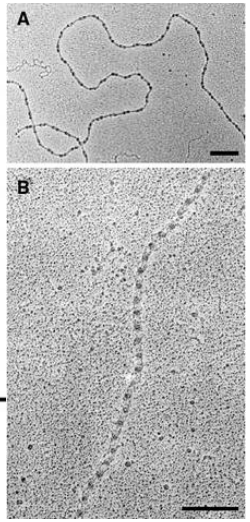


Fn7-10 alone,
40 kDa

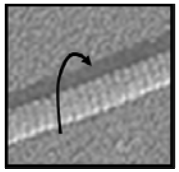
Still very usefull prior to cryo EM

Metal shadowing





- Aim :
  - Visualize the surface of particles
  - Handeness determination
- Technique :
  - Heavy atom evaporation
  - The sample is tilted in the evaporator



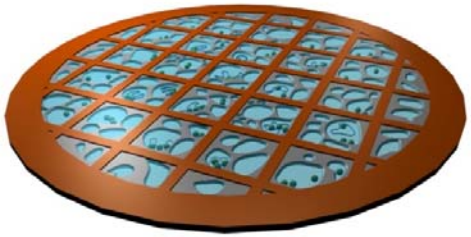
Negative staining and shadowing

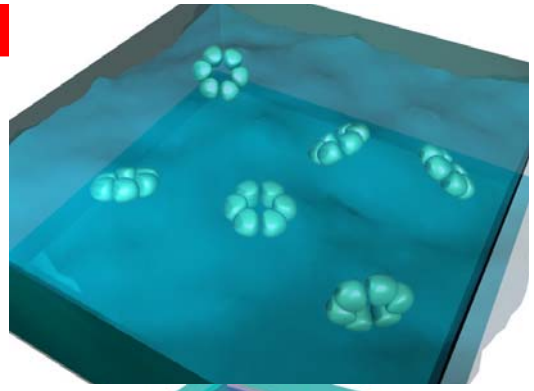
What we obtain

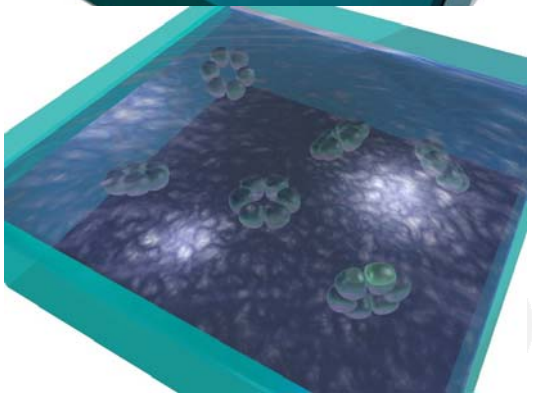
What we would like to have

To be close to native structure we need to preserve the hydrated state

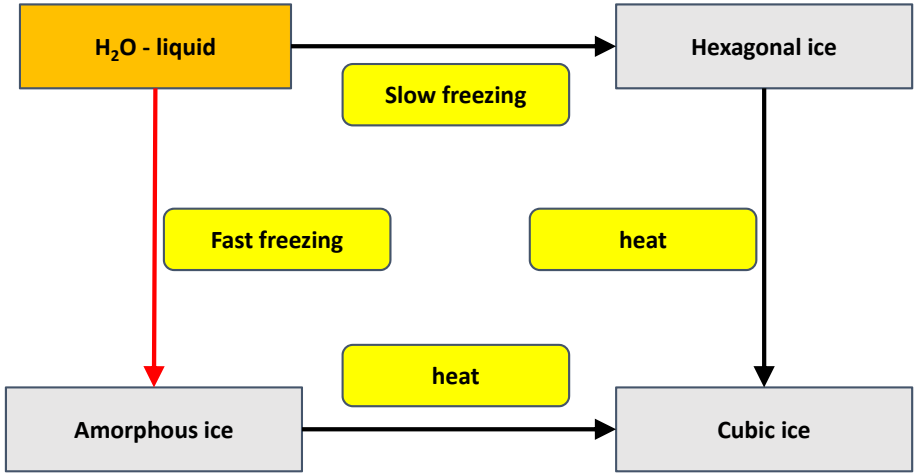
Cryo electron microscopy



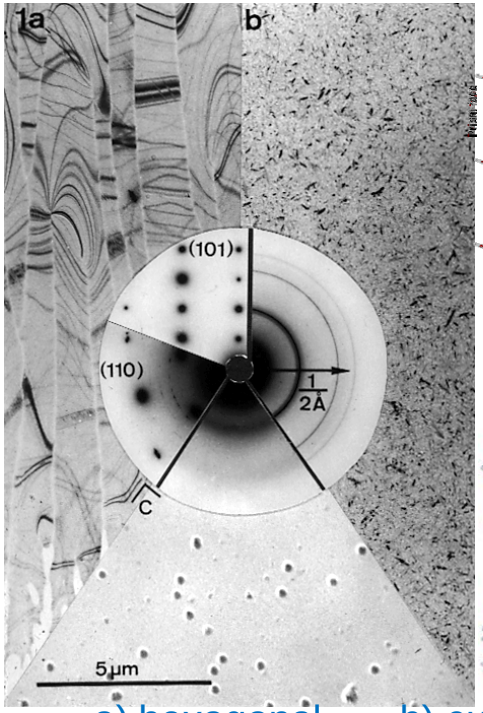


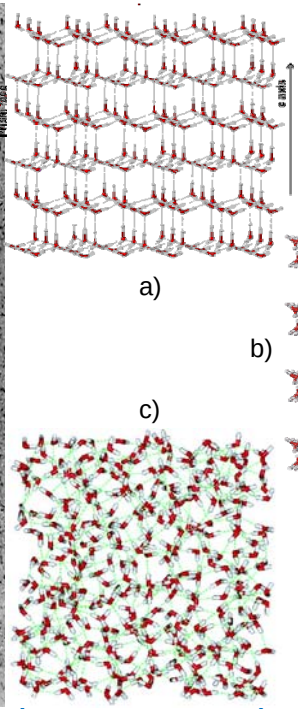


Cryo electron microscopy : Vitrification



Vitrification under atmospheric pressure over a thin tickness (<1µm)
Vitrification under high pressure 2000 bars up to 200 µm (cell tissues)

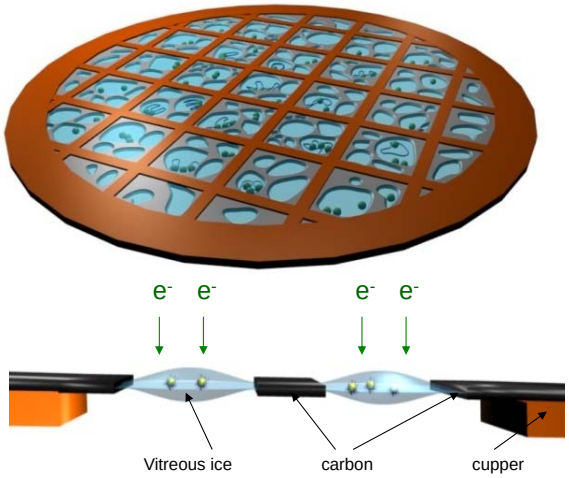


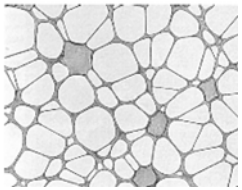


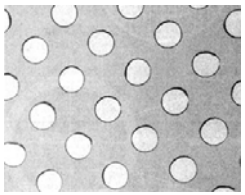
Dubochet et al., (1982)

a) hexagonal b) cubic c) amorphous

Cryo electron microscopy: Holey carbon grid

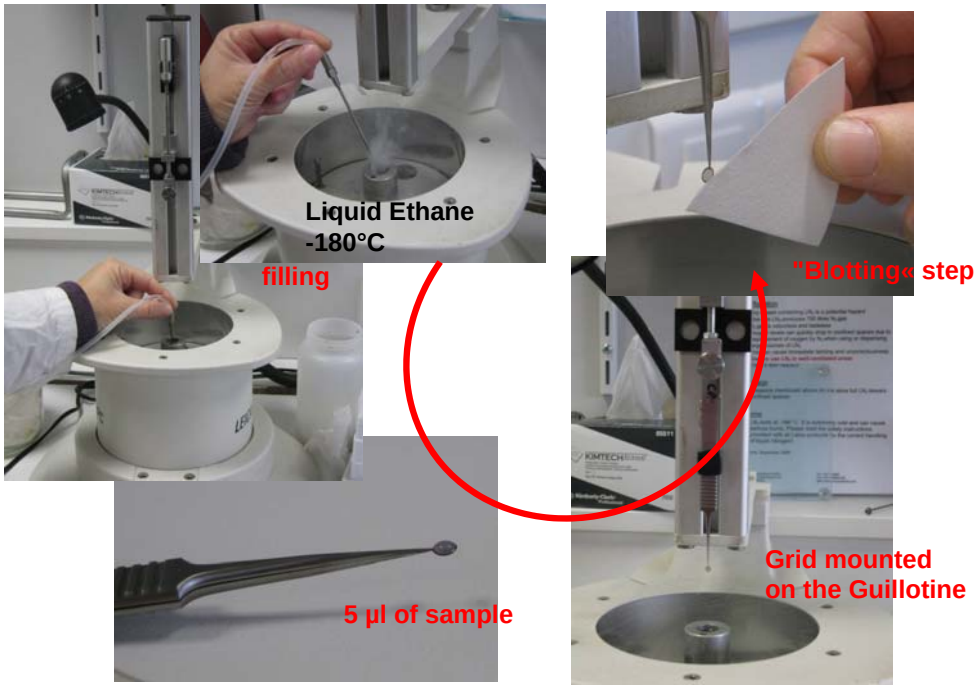


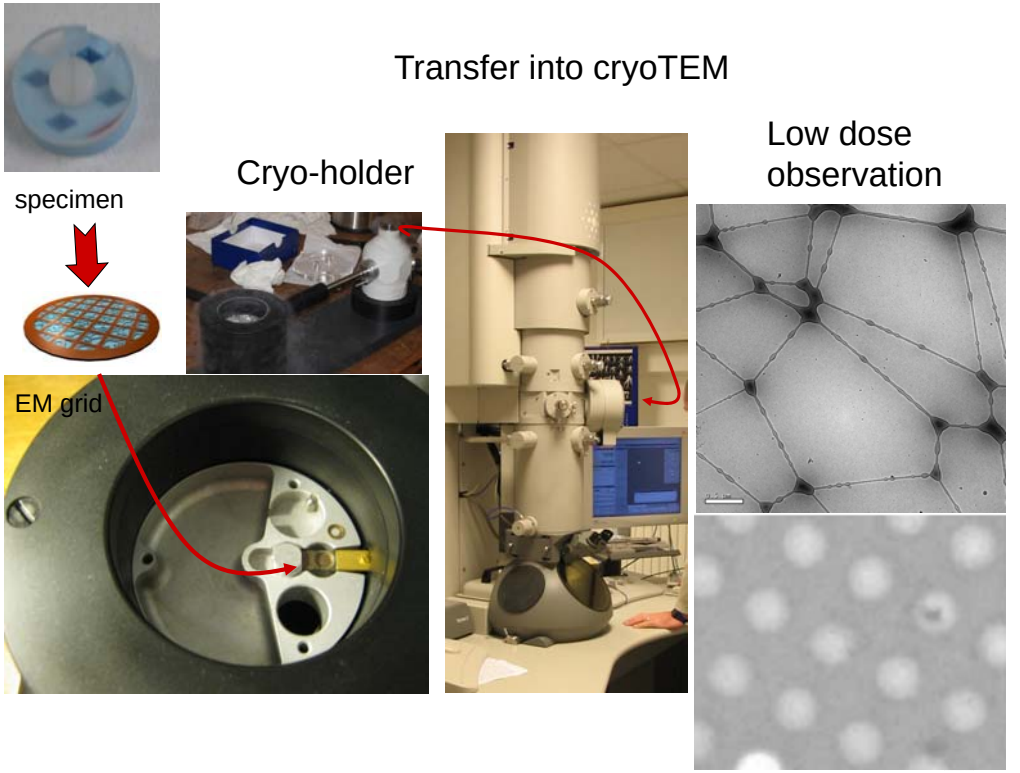




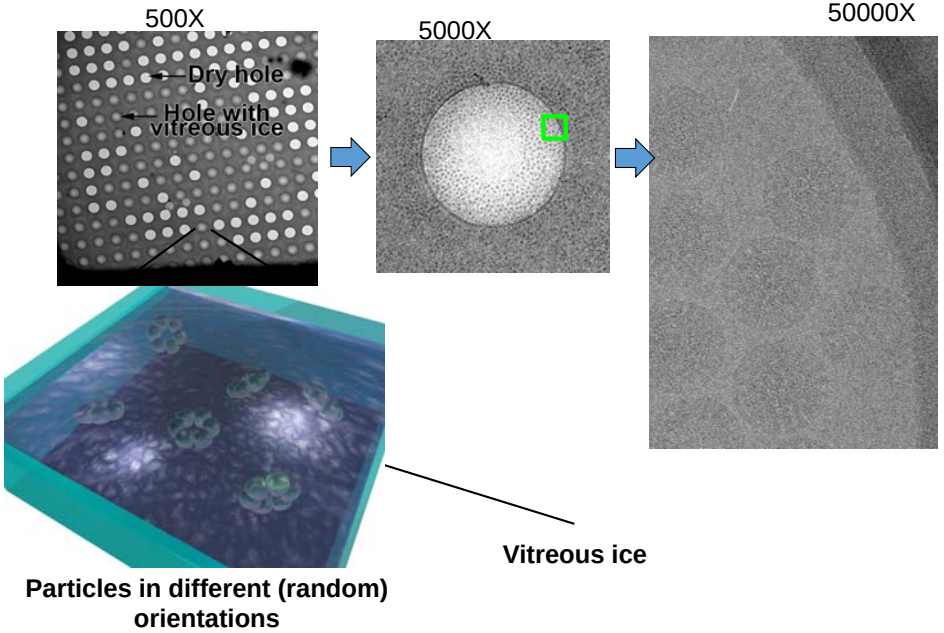
Cryo electron microscopy: Cryogenic fluids

cryogen	T fusion(K)	Tébulition(K)	T fusion (C)	T ébulition(C)	T (ébul - fus)
ethane	89,7	184,4	-183,45	-88,75	-94,7
helium	2,2	4,2	-270,95	-268,95	-2
hydrogen	14,1	20,4	-259,05	-252,75	-6,3
methane	90,5	109	-182,65	-164,15	-18,5
azote	63,1	77,3	-210,05	-195,85	-14,2
propane	83,3	230,9	-189,85	-42,25	-147,6





Cryo electron microscopy: What do you expect ?



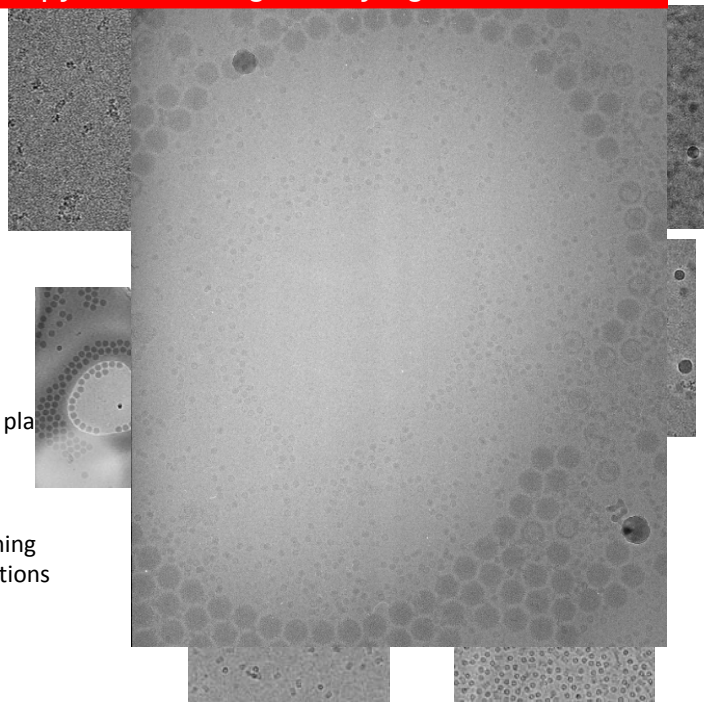
Cryo electron microscopy: What is a good cryo grid ?

Good amorphous ice
-not crystalline ice
-no « leopard skin »pattern
-no contamination

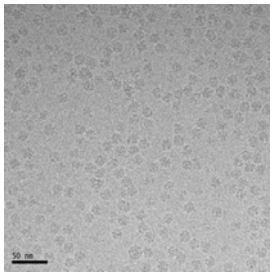
Appropriate ice thickness
-typically as thin as possible

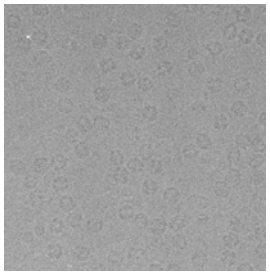
Clearly visible particles
-particle size and shape
-buffer composition
-defocus, movie mode, phase pla

Good particle distribution
-in holes
-dense but particles not touching
-randomly distributed orientations



Cryo electron microscopy: Advantages and drawbacks





Advantages :

hydrated state of the sample

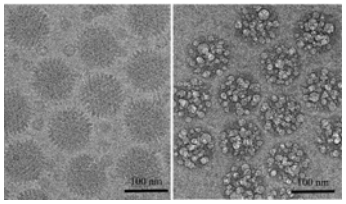
High resolution

Small amount of sample

Drawbacks :

Low contrast

Highly sensitive to electron dose

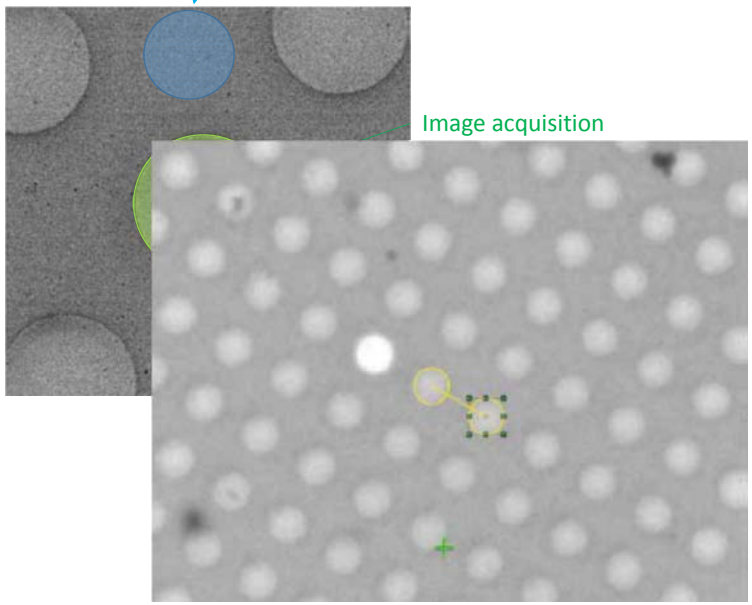


Cryo electron microscopy: Low dose acquisition

Low magnification image X 5,000

Settings (defocus, electron dose) at high magnification x50 000

Image acquisition



Cryo electron microscopy: Automated data acquisition

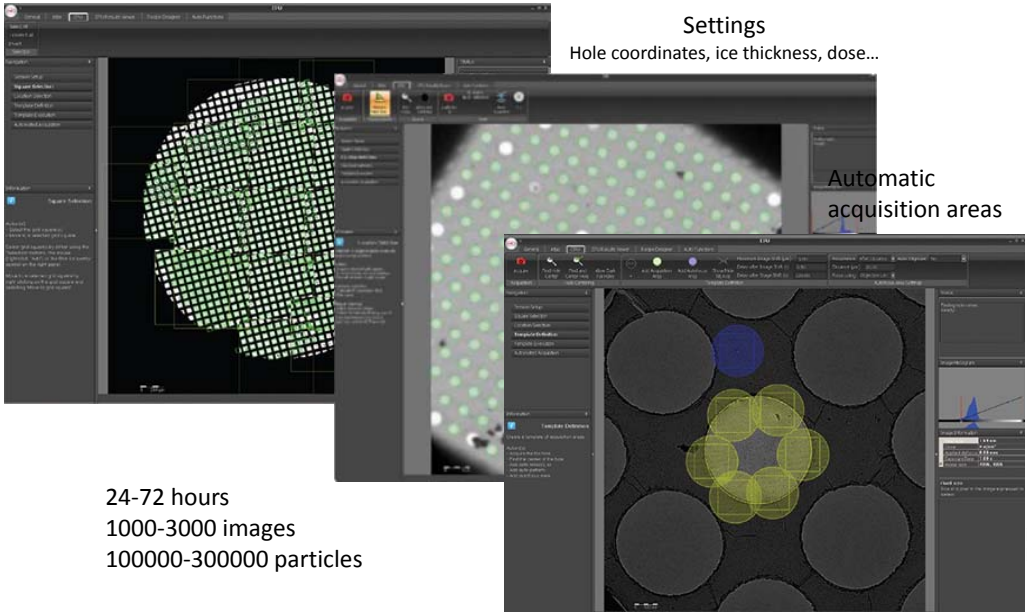
Example : Automated data acquisition software EPU (FEI)

Atlas = image of the EM grid

Settings

Hole coordinates, ice thickness, dose…

Automatic acquisition areas

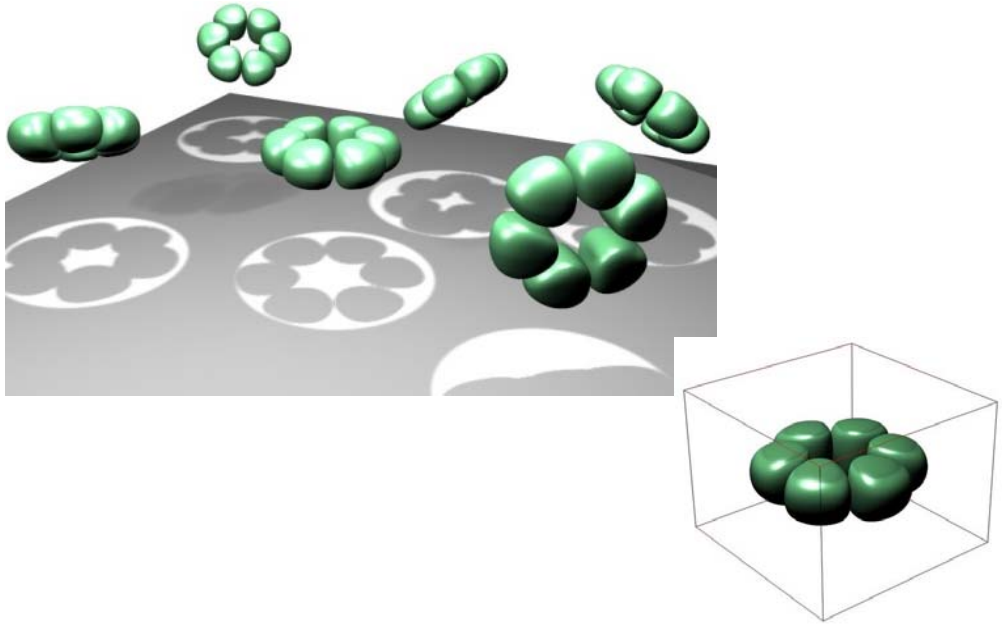


24-72 hours

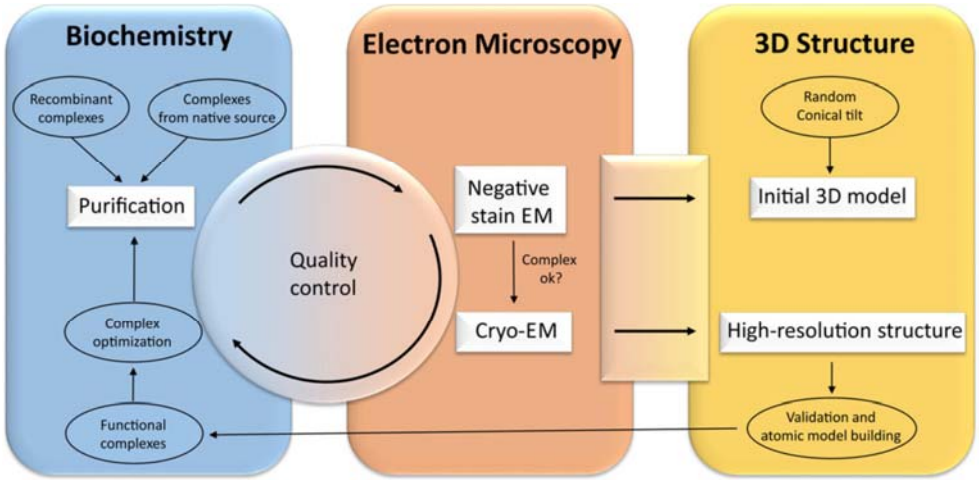
1000-3000 images

100000-300000 particles

Cryo electron microscopy: Projections suitable for 3D reconstruction



Cryo electron microscopy: Sample preparation workflow for high resolution structure determination

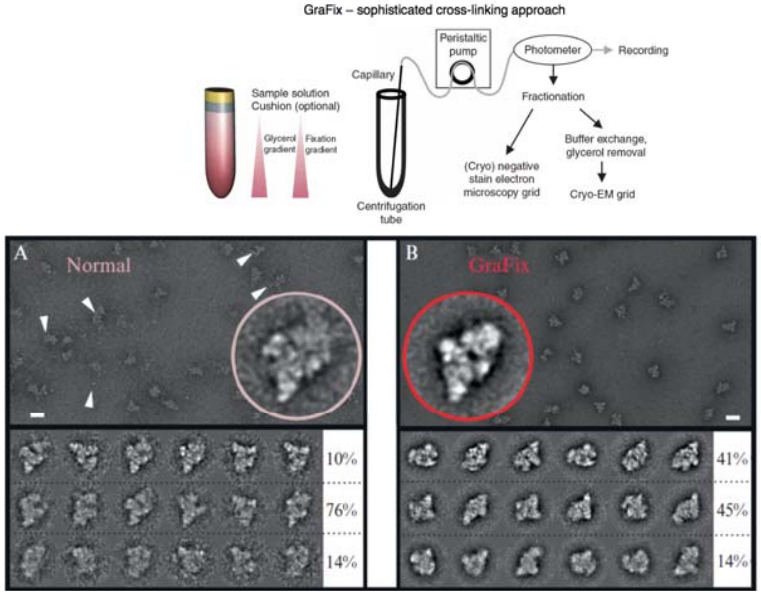


From Stark and Chari, 2015, Microscopy

1. Quality of purified protein (homogeneity, stability)
2. Distribution within the ice layer
3. Grid support

Improve the sample "stability"

Mild chemical fixation improves the stability of complex of protein (once deposited on the EM grid)



Kastner et al., (2008)
Nat. Methods 5:53-55.

Spliceosomes
in uranyl formate

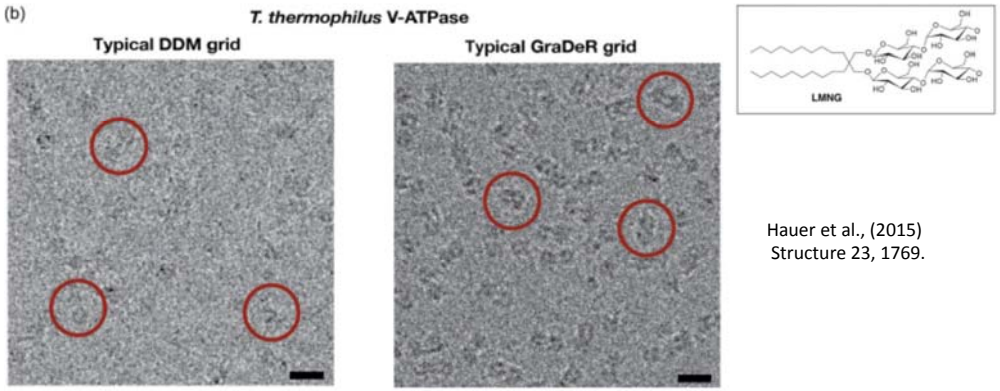
Improve the image "quality"

Optimize the buffer composition

-sample concentration
-buffer composition
-detergent

Beware of high concentrations of
-glycerol
-sugars
-salts
-detergents

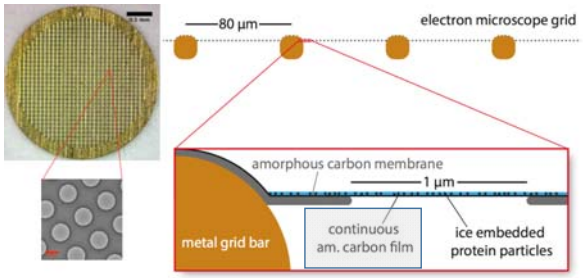
For Membrane protein, use lauryl maltose-neopentyl glycol and remove free detergent and micelle with Grafix

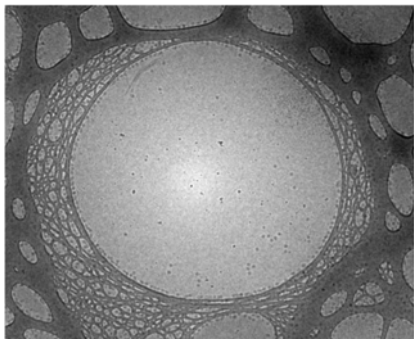


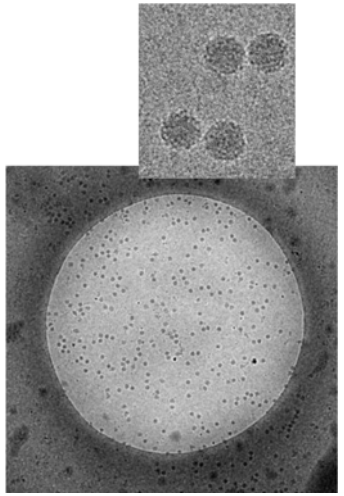
Hauer et al., (2015)
Structure 23, 1769.

Improve the sample distribution

Use an extra thin carbon support

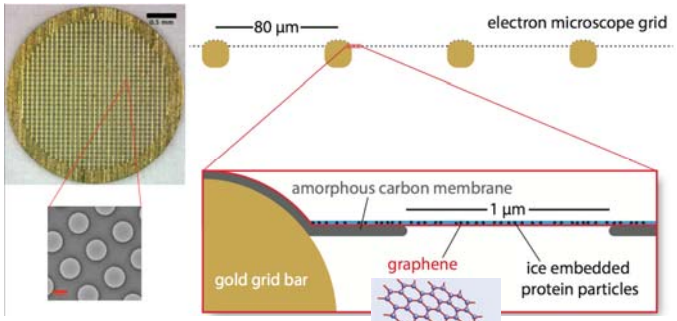






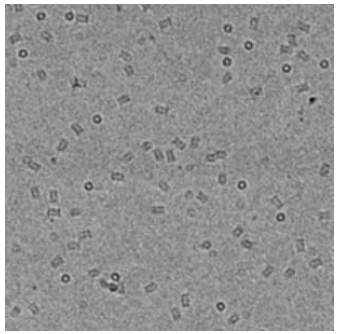
Improve the sample distribution

Use an extra graphene support

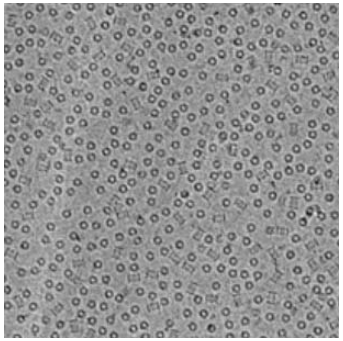


Russo & Passmore
Nature Methods 2014

Graphene modified with
low energy hydrogen plasma

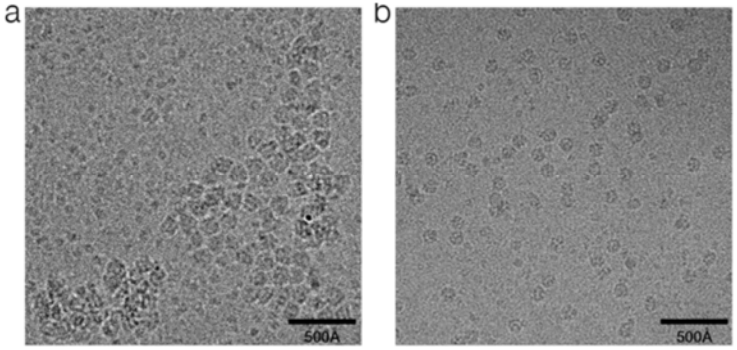


No Graphene



Use an extra graphene oxide support

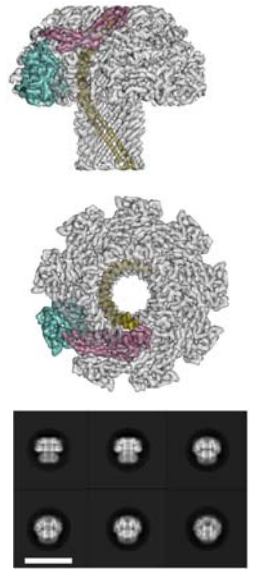
Specimen (3 ml of 0.2 mg/ml) was applied to holey-carbon grids overlaid with **graphene oxide** (without plasma treatment) and left to adhere for 30 s.



lysenin pore (~1 mg/mL) in pure ice with detergent micelles in the background. .

Lysenin pore on graphene-oxide

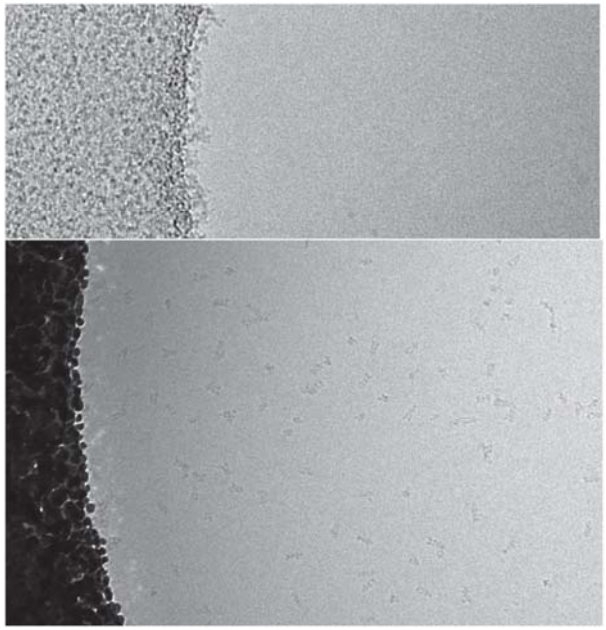
From Bokori-Brown et al, Nature Comm 2016

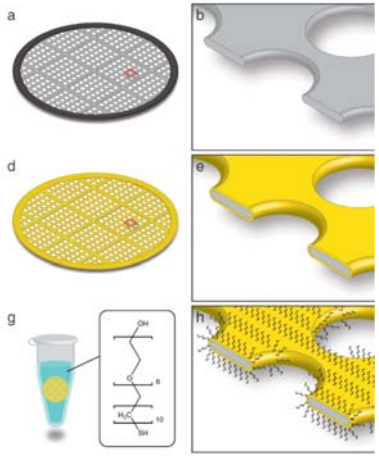


CryoEM reconstruction at 3.1 Å of a 315 kDa lysenin nonamer

Improve the sample distribution

Use self assembled monolayers on gold grid



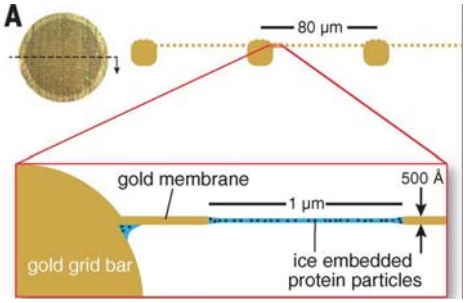


Meyerson Sci rep, 2014
Glutamate receptor

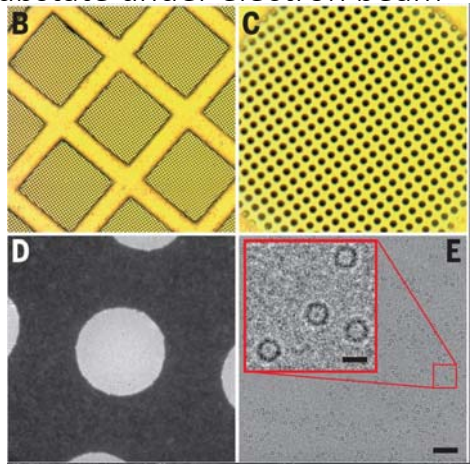
Improve the stability of the substate under electron beam

Use a gold grid

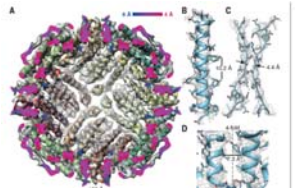
a gold specimen support
nearly eliminates substrate motion during irradiation



Russo & Passmore Science 2014



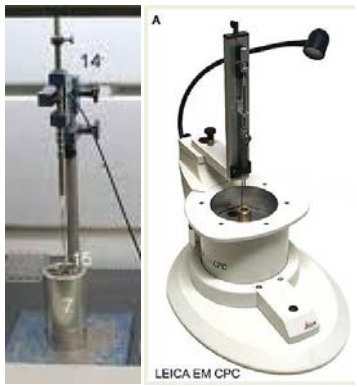
Compared with commercial am-C supports with nearly identical geometry, there was a 40-fold reduction movement. Apoferritin, 483 images , resolution 4.7 A.



Improve the sample distribution/stability

Plunging system

Home made



Control of the temperature and humidity

Gatan

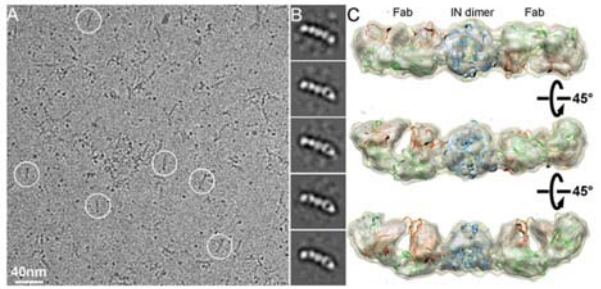
Leica

FEI

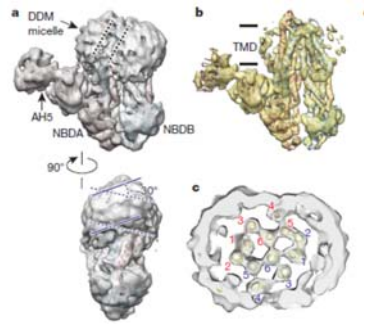
Freezing
-blotting time
-single or double side blotting
-waiting time
-multiple sample application

Particle size for cryoEM

Size > 500 kDa ; 200 kDa currently for near-atomic resolution
Symmetry: improves a lot !
Globular better than extended



HIV-1 integrase dimer (65 kDa) complexed with two Fabs (total of 165 kDa). 10 A resolution. Wu et al., 2012 Structure



ABC transporter TmrAB dimer (135 kDa) complexed with Fabs at 8.2 A resolution. Kim et al., 2015 Nature

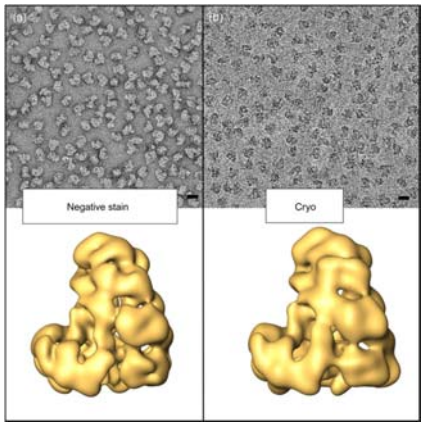
Start negative staining, then move to cryo

Get the more homogenous sample!

A lot of parameters can be changed

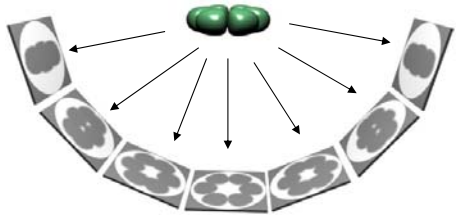
Start with the easiest one

If it is not working then try more sophisticated !

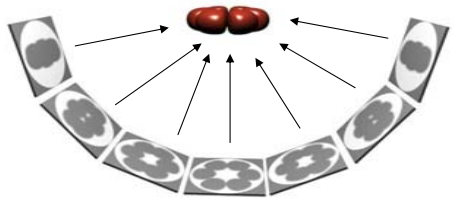


From Stark and Chari, 2015, Microscopy

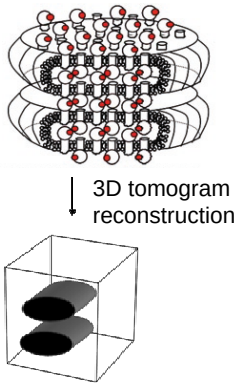
Cryo electron tomography: Sample which is not suitable for single particle analysis



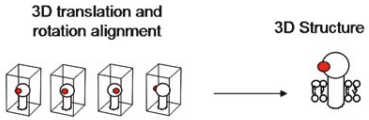
Cryo-electron tomography

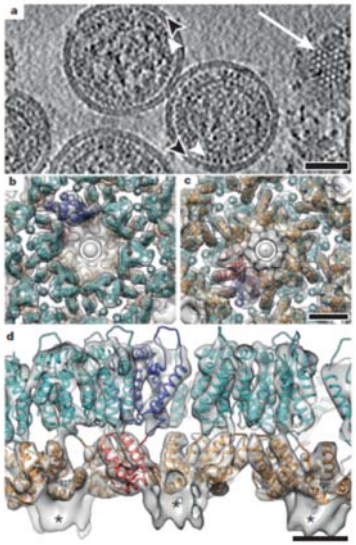


Cryo electron tomography: Sample which is not suitable for single particle analysis



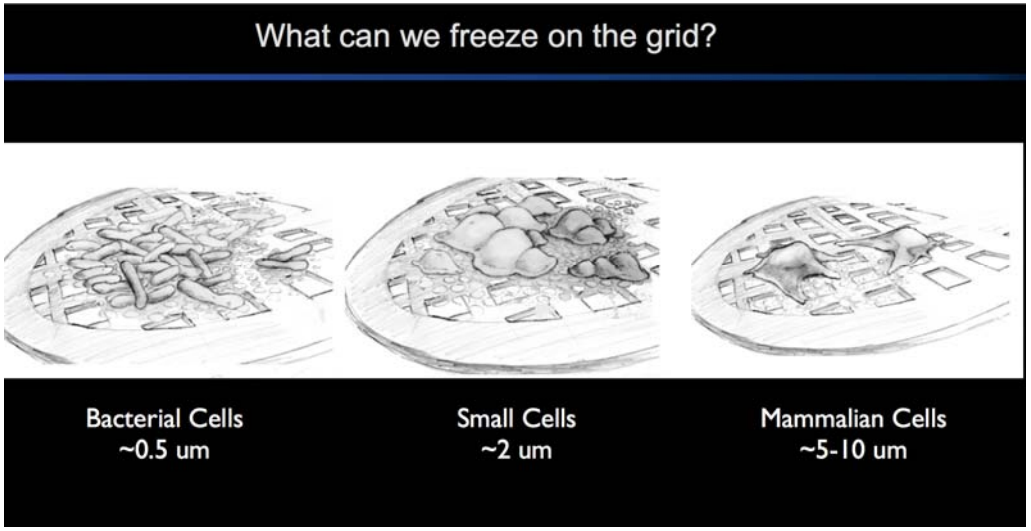
SUBTOMOGRAM AVERAGING





Structure of the immature HIV-1 capsid in intact virus particles at 8.8 Å resolution
Schur et al., Nature 2015

Cryo electron tomography on thick samples



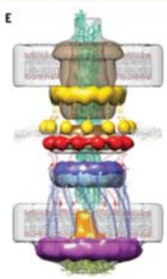
Cryo electron tomography on bacteria

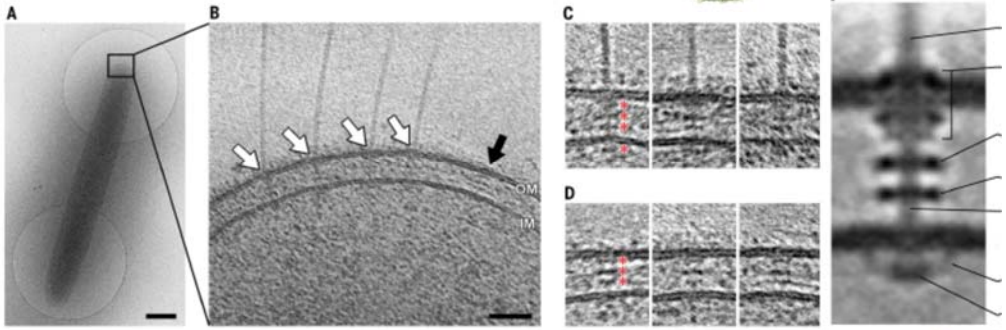
BACTERIAL PILI

Architecture of the type IVa pilus machine

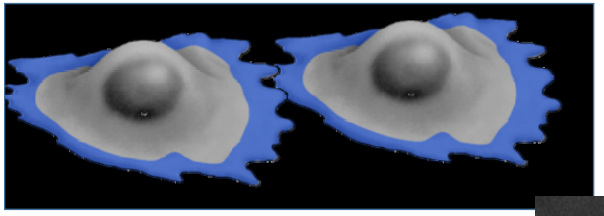
Yi-Wei Chang,[1,2] Lee A. Rettberg,[3] Anke Treuner-Lange,[3] Janet Iwasa,[4] Lotte Søgaard-Andersen,[3] Grant J. Jensen[1,2]*

Science 2016

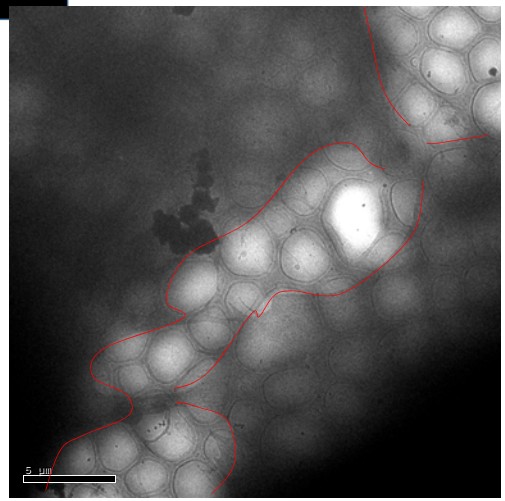




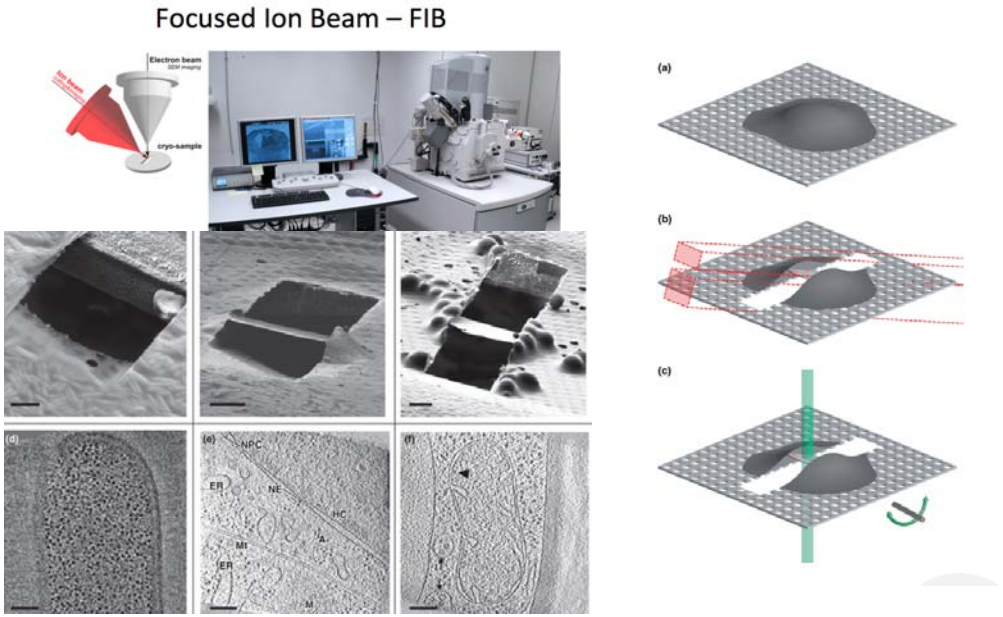
Cryo electron tomography on cells grown on EM grid



Edges of the cell suitable for CET



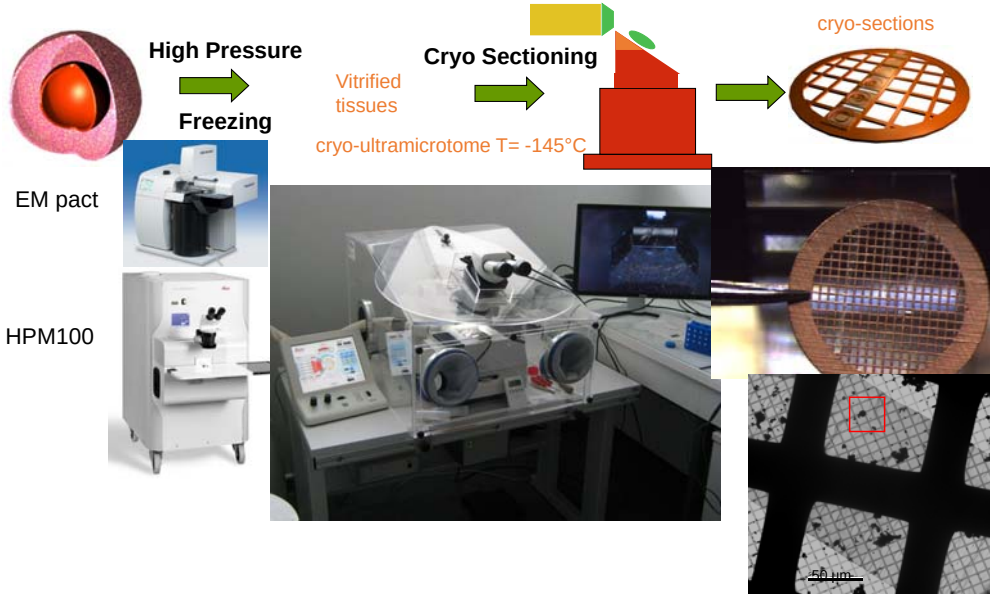
Cryo electron tomography on the ticker parts



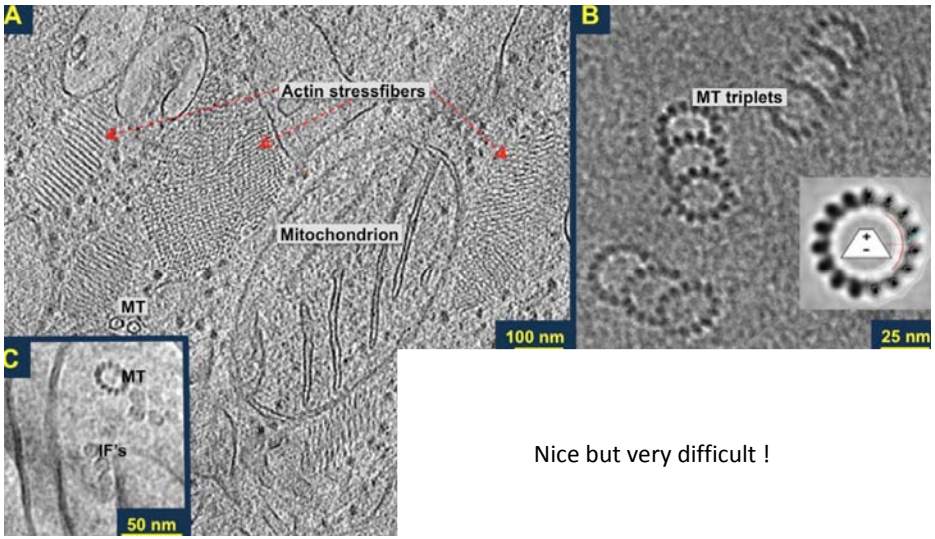
(d) tomogram of an E. coli cell. (e) Tomogram of an MDCK cell. (f) Tomogram of a mitochondria of a yeast cell . Villa et al , Cur op Struct Biol, 2013

Cryo electron tomography on tissues or pellet

CEMOVIS: Cryoelectron microscopy of vitreous section (40-100 nm thick) Dubochet and coll 2004



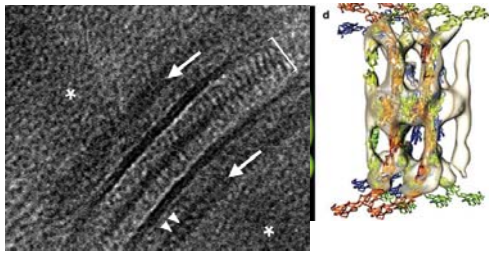
Examples of Cemovis



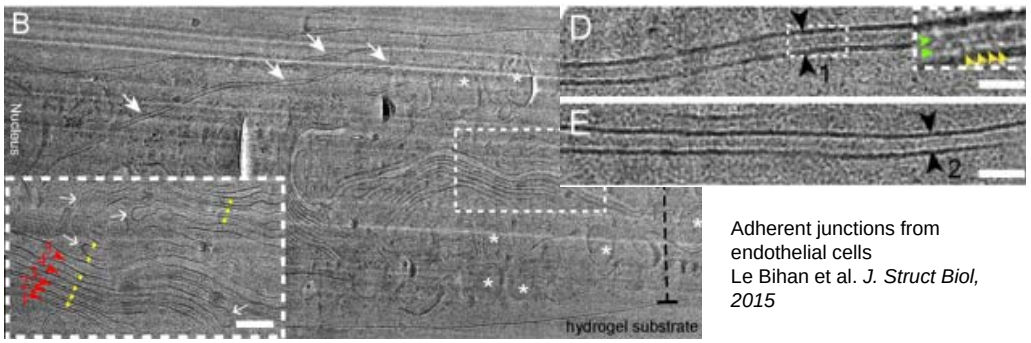
Nice but very difficult !

Hoenger A, & Bouchet-Marquis C. (2011) Cellular tomography. Adv Protein Chem Struct Biol.

Example of Cemovis



Desmosome from skin
Al-Amoudi et al. *Nature 2007, PNAS, 2010*

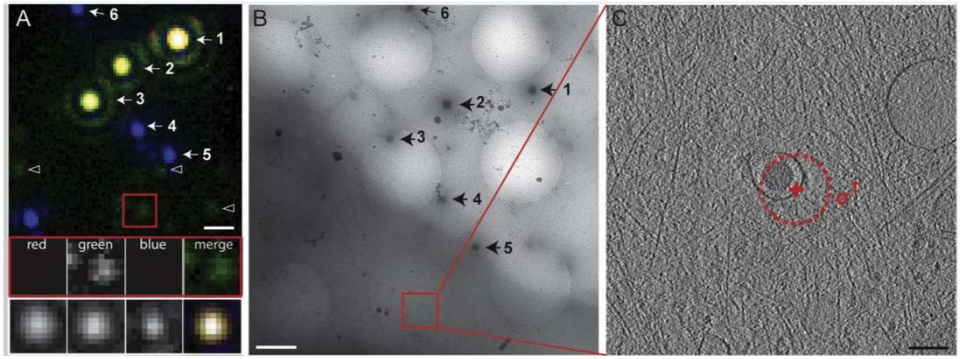


Adherent junctions from endothelial cells
Le Bihan et al. *J. Struct Biol, 2015*

Correlative light electron microscopy

Locate an event by light microscopy and then find the same event at higher mag using cryoEM (or EM)

Use a probe visible by EM (electron dense) and fluorescent microscopy



Schellenberger et al, ultramicroscopy 2014

The sample preparation step is crucial (as well as the purification and data collection)

other techniques exist for sample preparation:

For 2D crystal

Cryo-negative staining

Etc…

Questions????